

## Article

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# Microbially mediated reduction of Fe<sup>III</sup> and As<sup>V</sup> in Cambodian sediments amended with <sup>13</sup>C-labelled hexadecane and kerogen

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**Environmental context.** The use of groundwater with elevated concentrations of arsenic for drinking, cooking or irrigation has resulted in the worst mass poisoning in human history. This study shows that organic compounds that can be found in arsenic rich subsurface sediments may be used by indigenous microorganisms, contributing to the release of arsenic from the sediments into the groundwater. This study increases our understanding of the range of organic substrates (and their sources) that can potentially stimulate arsenic mobilisation into groundwaters.

**Abstract.** Microbial activity is generally accepted to play a critical role, with the aid of suitable organic carbon substrates, in the mobilisation of arsenic from sediments into shallow reducing groundwaters. The nature of the organic matter in natural aquifers driving the reduction of As<sup>V</sup> to As<sup>III</sup> is of particular importance but is poorly understood. In this study, sediments from an arsenic rich aquifer in Cambodia were amended with two <sup>13</sup>C-labelled organic substrates. <sup>13</sup>C-hexadecane was used as a model for potentially bioavailable long chain *n*-alkanes and a <sup>13</sup>C-kerogen analogue as a proxy for non-extractable organic matter. During anaerobic incubation for 8 weeks, significant Fe<sup>III</sup> reduction and As<sup>III</sup> mobilisation were observed in the biotic microcosms only, suggesting that these processes were microbially driven. Microcosms amended with <sup>13</sup>C-hexadecane exhibited a similar extent of Fe<sup>III</sup> reduction to the non-amended microcosms, but marginally higher As<sup>III</sup> release. Moreover, gas chromatography–mass spectrometry analysis showed that 65 % of the added <sup>13</sup>C-hexadecane was degraded during the 8-week incubation. The degradation of <sup>13</sup>C-hexadecane was microbially driven, as confirmed by DNA stable isotope probing (DNA-SIP). Amendment with <sup>13</sup>C-kerogen did not enhance Fe<sup>III</sup> reduction or As<sup>III</sup> mobilisation, and microbial degradation of kerogen could not be confirmed conclusively by DNA-SIP fractionation or <sup>13</sup>C incorporation in the phospholipid fatty acids. These data are, therefore, consistent with the utilisation of long chain *n*-alkanes (but not kerogen) as electron donors for anaerobic processes, potentially including Fe<sup>III</sup> and As<sup>V</sup> reduction in the subsurface.

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## Introduction

Arsenic release from sediments into groundwaters used for irrigation and drinking in West Bengal and Bangladesh has led to ‘the worst mass poisoning in human history’.<sup>[1]</sup> However, this problem is more extensive, as arsenic affected aquifers have been reported globally, from Cambodia and Vietnam,<sup>[2–6]</sup> to China,<sup>[7]</sup> Chile,<sup>[8]</sup> the USA<sup>[9]</sup> and Europe.<sup>[10]</sup> Owing to the catastrophic effect of groundwater arsenic pollution on the health of millions of people around the world, the factors controlling arsenic release into groundwater have been the subject of numerous studies, which have shown that various processes can contribute to increased As<sup>III</sup> mobilisation.<sup>[11–18]</sup>

One of these processes, microbial reduction of As<sup>V</sup>, is widely accepted as being an important mechanism of As<sup>III</sup> release in shallow reducing aquifers.<sup>[19–23]</sup> Bioavailable organic carbon serves both as a carbon source and electron donor for the microbial respiratory reduction of sedimentary As<sup>V</sup>, which serves as an electron acceptor, resulting in the release of As<sup>III</sup>, which is more mobile, to the aqueous phase.<sup>[20,24]</sup> As<sup>V</sup> reduction was shown repeatedly in a series of microcosm studies set up with sediments from arsenic affected aquifers, where simple organic proxies such as acetate or lactate were used to stimulate microbial mobilisation of arsenic.<sup>[15,19,20,22,25,26]</sup> Molecular studies in some of these systems revealed the presence of known

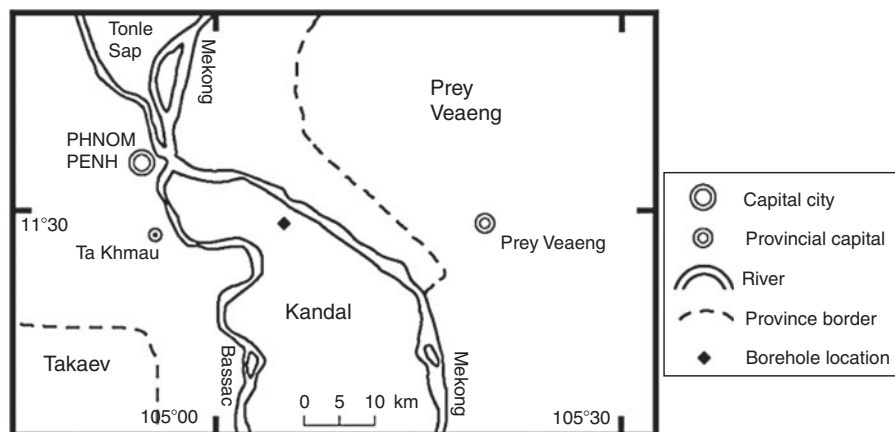


Fig. 1. Map of Kandal province, Cambodia, showing the location of the sampling borehole.

$\text{Fe}^{\text{III}}$  and  $\text{As}^{\text{V}}$  respiring bacteria, as well as arsenate respiratory reductase gene sequences.<sup>[19,25–27]</sup>

Nevertheless, the identification of the active members of the microbial communities in these complex sedimentary environments is challenging, especially when investigating specific microbe–substrate interactions. Thus, in some studies, the use of  $^{13}\text{C}$ -labelled organic carbon substrates (mainly acetate) in combination with molecular microbiology techniques (DNA stable isotope probing (DNA-SIP), followed by DNA fractionation and 16S rRNA gene cloning and sequencing) have allowed the identification of the active members of the microbial community during  $\text{As}^{\text{III}}$  mobilisation.<sup>[25,26,28]</sup> Alternatively to DNA-SIP, the incorporation of  $^{13}\text{C}$  in phospholipid fatty acids (PLFAs), which are the major components of the cell membranes and remain intact in viable cells,<sup>[29]</sup> can be used to investigate environmental microbial communities.<sup>[30]</sup> This is because the concentration and distribution of PLFAs has the capacity to reflect rapid changes in the microbial populations and link specific biomarkers to a phylogenetic or functional group of organisms.<sup>[30–32]</sup>

Despite the usefulness of previous microcosm studies that have focussed on simple organic substrates (acetate or lactate), it is known that sediments in arsenic affected aquifers are often organically lean, i.e. with less than 1% organic matter (OM)<sup>[33–35]</sup> and they contain different types of OM. Recent studies in sediments from arsenic hot spots in West Bengal,<sup>[27,36]</sup> Cambodia,<sup>[22,37]</sup> and Taiwan<sup>[38]</sup> have shown that extractable OM, including long chain *n*-alkanes and other hydrocarbons of immature (e.g. petroleum) origin are present. Microbial degradation of *n*-alkanes has been shown previously for a range of microorganisms (pure isolates or mixed microbial communities) under aerobic and anaerobic environments.<sup>[39–42]</sup> Moreover, a recent microcosm study showed almost complete degradation of (naturally occurring) petroleum derived *n*-alkanes during incubation of arsenic-rich West Bengal sediments under anoxic, arsenic-reducing conditions,<sup>[27]</sup> suggesting that these hydrocarbons could play a role in mediating the release of  $\text{As}^{\text{III}}$ . However, extractable OM makes up ~5% of the sedimentary OM, meaning that the majority, ~95%, is part of the non-extractable material (e.g. kerogen<sup>[43]</sup>). To date, microbial degradation of kerogen appears largely restricted to well-oxygenated settings,<sup>[44]</sup> and is refractory under anaerobic conditions.<sup>[45]</sup> It remains unclear if kerogen can serve as a carbon source and electron donor for the microbial respiratory reduction of sedimentary  $\text{Fe}^{\text{III}}$  or  $\text{As}^{\text{V}}$ .

Therefore, the aims of this study were to investigate (i) whether environmentally relevant organic carbon substrates can be microbially degraded and (ii) to assess their affect on  $\text{As}^{\text{III}}$  mobilisation. We investigated these aims by establishing a series of anoxic microcosms, using sediments collected from an arsenic-affected aquifer in Cambodia, and supplemented with  $^{13}\text{C}$ -labelled substrates.  $^{13}\text{C}$ -Hexadecane was used as a representative of long chain *n*-alkanes, which are part of the extractable OM (i.e. the part of the OM that can be extracted using common organic solvents). We also artificially matured  $^{13}\text{C}$ -labelled algal biomass to generate a labelled kerogen analogue to represent the non-extractable part of the OM. Following anaerobic incubation for 8 weeks, degradation of the  $^{13}\text{C}$ -labelled substrates was determined by gas chromatography–mass spectrometry (GC-MS), and their incorporation into the microbial biomass was detected by DNA-SIP fractionation. This study should provide a better understanding of the mechanisms underpinning microbial release of  $\text{As}^{\text{III}}$  in natural, arsenic affected aquifers.

## Methodology

### Study area and sample collection

The sampling site was located in Rotaing village (Kean Svay District, Kandal Province), which lies between the Bassac and Mekong rivers, south of Phnom Penh, in Cambodia (Fig. 1). This region is known for high arsenic groundwater concentrations.<sup>[2,5,46]</sup> The sediment sample of this study was taken from a Holocene clay-rich horizon (6–7 m below ground surface) from a shallow aquifer, which is characterised by elevated ( $>50 \mu\text{g L}^{-1}$ ) groundwater arsenic concentrations.<sup>[4,5,47]</sup> The sediment was collected in July 2008 by drilling fluid-less within 1 m of sample collection and then up to 7 m, by augering, using groundwater from a nearby anoxic ( $>20\text{-m}$  depth) well as a drilling fluid. Upon retrieval, the full intact cores were immediately transferred into a glove bag, which was continuously flushed with  $\text{N}_2$  following the methods recommended by Rowland et al.<sup>[48]</sup> Sub-samples were sent to the UK, where they were stored under anoxic conditions, at  $4^\circ\text{C}$  in the dark.

### Sediment characterisation

Total carbon values were determined using a EuroVector EA3000 CHN elemental analyser (EuroVector, Milan, Italy). The inorganic carbon content was determined using a Strohllein Coulomat 702 analyser (Markus de Vries, Schwabach,

Germany), which was modified to use phosphoric acid to release carbon dioxide from inorganic carbon in the sample. Total organic carbon (TOC) was calculated by subtracting inorganic carbon from total carbon values. Concentrations of the major and trace elements were determined on air-dried, finely ground pressed powder briquettes by X-ray fluorescence spectrometry (XRF; Axios, PANalytical, Almelo, Netherlands).

### <sup>13</sup>C-Hexadecane and <sup>13</sup>C-kerogen

<sup>13</sup>C-Hexadecane with certified label incorporation of 98.9% was purchased from Buchem (Buchem BV, Apeldoorn, the Netherlands). <sup>13</sup>C-Kerogen with label incorporation of 98% was produced by artificial maturation of <sup>13</sup>C-labelled cyanobacterial biomass purchased from Cambridge Isotope Laboratories (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA). The detailed maturation procedure and characterisation of the <sup>13</sup>C-labelled kerogen obtained is described elsewhere.<sup>[49]</sup> In short, <sup>13</sup>C-labelled biomass was crushed in liquid N<sub>2</sub>, powdered and artificially matured in sealed gold cells at 330 °C and 300 bar for 24 h in the absence of water. The gold capsules were cracked and all the material was rinsed out using a combination of dichloromethane (DCM) and methanol (MeOH), both supplied by Sigma-Aldrich (Gillingham, UK). The kerogen was separated from the bitumen by sequential sonic extraction with DCM, DCM-MeOH and MeOH. Analyses by pyrolysis-gas chromatography-mass spectrometry indicates a good resemblance with pyrolysates of natural kerogens.<sup>[49]</sup>

### Microcosm experiments and analyses

Four microcosm treatments were established in triplicate: (i) sediment only, (ii) sediment amended with 20 µL (2.3 mg g<sup>-1</sup> sediment) of <sup>13</sup>C-hexadecane, (iii) sediment amended with 0.5 mg (0.1 mg g<sup>-1</sup> sediment) of <sup>13</sup>C-kerogen and (iv) sediment sterilised by autoclaving (abiotic control). All microcosms were set up in 100-mL serum bottles (acid washed and sterilised by autoclaving), in a glove box under anaerobic conditions (98 : 2 %, N<sub>2</sub>/H<sub>2</sub> atmosphere). All microcosms were set up by mixing 10 g of sediment and 20 mL of anaerobic sterile synthetic groundwater,<sup>[22]</sup> apart from the <sup>13</sup>C-kerogen amended microcosms, which were set up by mixing 5 g of sediment and 10 mL of anaerobic sterile synthetic groundwater. The microcosms were maintained under anaerobic conditions by sealing the serum bottles with butyl rubber stoppers and aluminium crimp seals, and incubated at 20 °C in the dark. Approximately 2 mL of slurry was sampled anaerobically at the start of the incubation (*t* = 0), and after 2, 4, 6 and 8 weeks, with the exception of the kerogen amended microcosms, which were sampled at time points 0, 10 days and 8 weeks. Immediately after sampling, the pH and reduction potential (*E*<sub>h</sub>) in the slurry sample was measured, and the concentration of 0.5 M HCl extractable Fe<sup>II</sup> was determined using a ferrozine-based spectrophotometric assay.<sup>[50]</sup> In addition, arsenic speciation in the porewaters (filtered sub-samples through a 0.2-µm pore size filter) was determined by ion chromatography-inductively coupled plasma-mass spectrometry (IC-ICP-MS) using the method developed in Gault et al.<sup>[51]</sup> and described in Rowland et al.<sup>[22]</sup> Nitrate and sulfate measurements were taken in filtered slurry samples (through a 0.2-µm pore size filter) using a Metrohm 761 compact ion exchange chromatograph (Metrohm AG, Herisau, Switzerland). All microcosms were kept frozen at the end of their incubation period (8 weeks) until further analyses.

### DNA isolation and 16S rRNA gene amplicon pyrosequencing

DNA was extracted from a sediment sample before incubation, a slurry sample from the sediment only treatment at the end of the incubation period (*t* = 8 weeks) and a slurry sample from the treatment amended with <sup>13</sup>C-hexadecane at the end of the incubation period (*t* = 8 weeks). DNA isolations were carried out using the PowerSoil DNA extraction kit (MOBIO Laboratories, Carlsbad, CA, USA). Polymerase chain reaction (PCR) for amplicon pyrosequencing was performed using tagged fusion bacterial primers 27F and 338R, targeting the V1-V2 hypervariable region of the bacterial 16S rRNA gene (see Supplementary material). The pyrosequencing run was performed at The University of Manchester sequencing facility, using a Roche 454 Life Sciences GS Junior system. The 454 pyrosequencing reads were analysed using *Qiime 1.6.0* release.<sup>[52]</sup> Details about the primer design and the analysis of the obtained pyrosequencing reads can be found in the Supplementary material. The pyrosequencing reads of this study have been deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP046303 (BioProject ID: PRJNA260474).

### DNA-SIP

DNA-SIP was performed on the extracted DNA from the treatment amended with <sup>13</sup>C-hexadecane at the end of the incubation period (*t* = 8 weeks), according to the protocol by Neufeld et al.,<sup>[53]</sup> but adapted for 3.9-mL Quick-Seal, Polyallomer tubes (Beckman Coulter, Brea, CA, USA). Heavy and light DNA fractions were separated on a CsCl gradient, following centrifugation with an Optima TL-100 ultracentrifuge (Beckman Coulter) at 178 693g for 47 h at 20 °C. Eleven fractions of ~330-µL each were collected from each sample, and their density was determined using an analytical balance. The DNA in each of the collected fractions was precipitated as described previously,<sup>[53]</sup> resuspended in 30 µL of 10 mM Tris, 1 mM ethylenediamine-tetraacetic acid buffer, quantified using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA), and used as template for PCR amplification using primers 338F-GC and 519R,<sup>[54]</sup> targeting the hypervariable V3 region of the 16S rRNA gene. The PCR products were then subjected to denaturing gradient gel electrophoresis (DGGE) analysis, as previously described,<sup>[54]</sup> to investigate successful separation of the heavy (<sup>13</sup>C-labelled) and the light (unlabelled) DNA fractions.

### Lipid (n-alkane and PLFA) extraction, fractionation and analysis

Lipids were extracted from 10 g of the original sediment amended with 20 µL of <sup>13</sup>C-hexadecane (representing the *t* = 0 of the 'sediment + <sup>13</sup>C-hexadecane' treatment), and from the remaining sediment slurries of the 'sediment + <sup>13</sup>C-hexadecane' and 'sediment + <sup>13</sup>C-kerogen' treatments at the end of the 8-week incubation period. The sediments (or slurries) were freeze-dried and ground, and the total lipid extracts (TLEs) were obtained by Bligh-Dyer extraction using a single-phase mixture of chloroform-methanol-KH<sub>2</sub>PO<sub>4</sub> buffer (1 : 2 : 0.8, v/v/v, 18 mL).<sup>[55]</sup> After centrifugation at 2500 rpm for 15 min, the TLEs were concentrated using rotary evaporation. Subsequently, aliquots of the TLEs were separated into three fractions using a column packed with activated silica gel (0.5 g, 60-Å particle size). These fractions were eluted with chloroform-acetic acid (100 : 1, v/v, 5 mL; 'simple lipid fraction'), acetone (20 mL; 'glycolipid fraction') and methanol (5 mL; 'phospholipid (PL)



**Table 1.** Amounts and molarities of potential electron acceptors and donors per gram of sediment

| Electron donor or acceptor | Initial amounts<br>(mg g <sup>-1</sup> sediment) | Reduced or oxidised<br>(μmoles g <sup>-1</sup> sediment) | Comment                                 |
|----------------------------|--|--|---|
| Fe                         | 56   | 112  | 11.2 % of the total Fe                  |
| As                         | 0.014  | 0.00088  | 0.47 % of the total As                  |
| Nitrate                    | 0.003  | up to 0.048  | But no clear nitrate reduction detected |
| Sulfate                    | 0.01   | up to 0.164  | But no clear sulfate reduction detected |
| TOC                        | 9.7  | ?  | Unidentified sedimentary OM             |
| Hexadecane                 | 1.1  | 2.6  | Each molecule contains 16 C atoms       |

fraction'). The simple lipid fraction was further separated into two fractions using a column packed with (activated) Al<sub>2</sub>O<sub>3</sub> by elution with hexane–dichloromethane (9 : 1, v/v, 3 mL; 'hydrocarbon fraction') and dichloromethane–methanol (1 : 1, v/v, 3 mL; 'polar fraction').<sup>[22]</sup> PLFAs were generated and analysed as described in the Supplementary material.

## Results

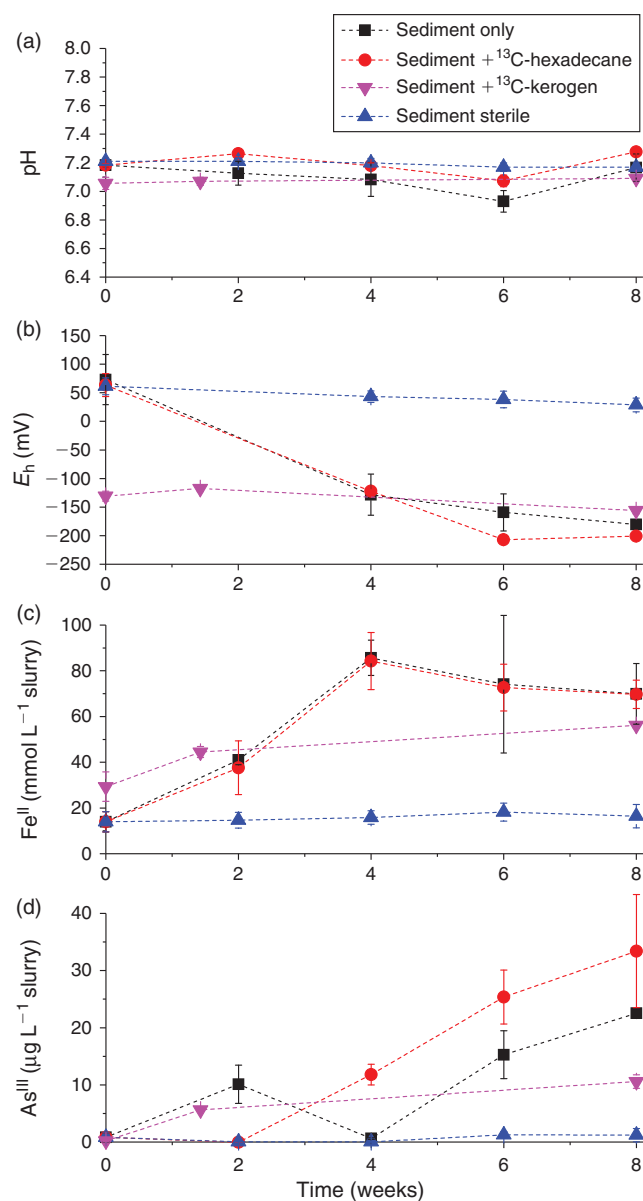
### Sediment characteristics

The total organic carbon (TOC) content in the Cambodian sediment was 0.97 % (9.7 mg TOC g<sup>-1</sup> of sediment), whereas the concentrations of total Fe, Mn and As were 56 000, 880 and 14 μg g<sup>-1</sup> of sediment, as determined by XRF analysis (Table 1).

### Fe<sup>III</sup> reduction and mobilisation of As<sup>III</sup>

Four microcosm treatments were set up to investigate the role of OM in microbially mediated arsenic mobilisation processes. During the 8-week incubation, the pH, the *E<sub>h</sub>* and the concentrations of Fe<sup>II</sup> and As<sup>III</sup> were monitored (Fig. 2). The pH in all microcosms remained near neutral during the incubation period, with average values ranging between 6.9 and 7.3 (Fig. 2a). The starting *E<sub>h</sub>* in the kerogen amended microcosms was significantly lower than in the other microcosms (−130 mV compared to between +43 and +73 mV), possibly because these microcosms were set up at a later time point than the other microcosms and the sediment may have become more reducing. After anaerobic incubation for 8 weeks, the *E<sub>h</sub>* in the biotic microcosms decreased gradually to values between −156 and −200 mV but in the sterile microcosms it remained at positive values (Fig. 2b).

The concentration of Fe<sup>II</sup> in the 'sediment only' and 'sediment + <sup>13</sup>C-hexadecane' treatments exhibited similar patterns, increasing from 14 mmol Fe<sup>II</sup> L<sup>-1</sup> slurry at the start of the experiment to 70 mmol L<sup>-1</sup> by the end of the incubation period (8 weeks), with the highest increase observed during the first 4 weeks (Fig. 2c). The reduction of up to 56 mM Fe<sup>III</sup> during the incubations corresponds to 112 μmoles of Fe<sup>III</sup> g<sup>-1</sup> of sediment or 6.272 mg g<sup>-1</sup> of sediment (i.e. 11.2 % of the total sedimentary Fe was reduced, Table 1). In the microcosms amended with <sup>13</sup>C-kerogen, there was a lower increase in Fe<sup>II</sup> concentration, from 29 to 56 mmol Fe<sup>II</sup> L<sup>-1</sup>. The reduction of 27 mmol L<sup>-1</sup> corresponds to 54 μmoles of Fe<sup>III</sup> g<sup>-1</sup> of sediment or 3.024 μg g<sup>-1</sup> of sediment. In the abiotic (autoclaved) microcosms, the concentration of Fe<sup>II</sup> remained stable during the incubation period. For comparison, concentrations of other competing electron acceptors such as nitrate and sulfate were



**Fig. 2.** Measurement of (a) pH, (b) reduction potential (*E<sub>h</sub>*) (mV), (c) Fe<sup>II</sup> concentrations (mmol L<sup>-1</sup> slurry) and (d) As<sup>III</sup> concentrations (μg L<sup>-1</sup> porewaters), in anaerobic microcosms set up with arsenic rich Cambodian sediment and incubated for 8 weeks. Measurements were taken on three microcosm replicates and the error bars represent standard deviations from the mean.

very low during the incubations, respectively up to 1.5 and 7 mg L<sup>-1</sup>. These concentrations were close to our analytical detection limits. Thus it was not possible to establish any conclusive nitrate- or sulfate-reducing trends during the course of the experiment (data not shown). These maximum concentrations correspond to 0.048  $\mu\text{mol}$  of NO<sub>3</sub><sup>-</sup> and 0.146  $\mu\text{mol}$  of SO<sub>4</sub><sup>2-</sup> g<sup>-1</sup> of sediment (Table 1).

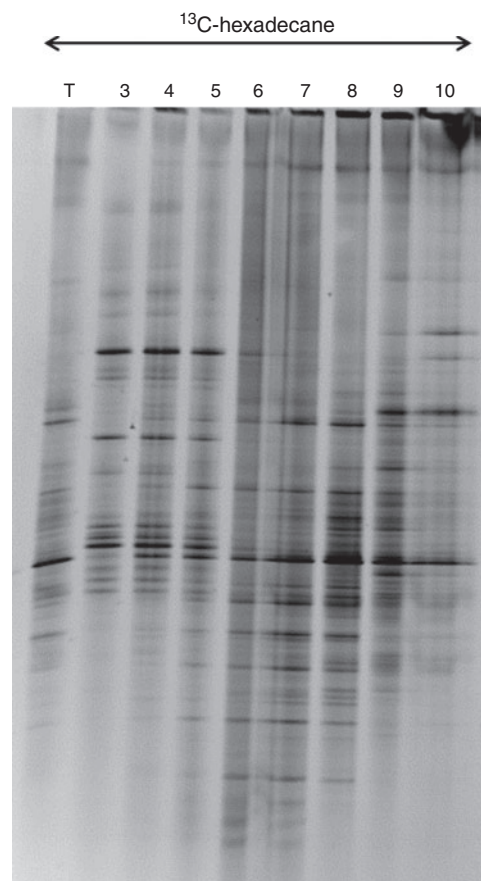
Compared to background As<sup>III</sup> concentrations of 0.19–0.83  $\mu\text{g}$  L<sup>-1</sup> at the start of the incubations ( $t = 0$ ), there was significant release of As<sup>III</sup> into the porewaters of all biotic microcosms after anaerobic incubation for 8 weeks, as the concentrations of As<sup>III</sup> increased to 23, 33 and 11  $\mu\text{g}$  L<sup>-1</sup> in the respective ‘sediment only’, ‘sediment + <sup>13</sup>C-hexadecane’ and ‘sediment + <sup>13</sup>C-kerogen’ treatments (Fig. 2d). The reduction of up to 33  $\mu\text{g}$  As L<sup>-1</sup> corresponds to 0.066  $\mu\text{g}$  As g<sup>-1</sup> of sediment (i.e. 0.47 % of the total As was reduced) or 0.00088  $\mu\text{mol}$  of As g<sup>-1</sup> of sediment (Table 1). In contrast, the concentration of As<sup>III</sup> in the abiotic microcosms (‘sediment sterile’) remained stable throughout the 8-week incubation period, and up to 1.8  $\mu\text{g}$  L<sup>-1</sup>.

#### Bacterial communities assessed by 16S rRNA gene amplicon pyrosequencing

Following 16S rRNA gene amplicon pyrosequencing, more than 2000 reads were retrieved from each sample and 125 to 188 operational taxonomic units were identified at 97 % sequence ID similarity level (Table S1 in the Supplementary material). Sequence analyses indicated a complex background community dominated by organisms affiliated with *Sideroxydans* species of the Gallionellaceae family. This could be a result of the long storage of the sediment before the set up of the microcosms of this study (20 months). However, compared to the microbial community before incubations ( $t = 0$ , sediment only), anaerobic incubation of the sediments (with or without supplemented <sup>13</sup>C-hexadecane) led to a clear enrichment of sequences related to *Geobacter* and *Sulfurospirillum* genera (Table S2). Abundance of *Geobacter*-related sequences almost doubled from 3.6 % of the initial bacterial community (before incubations) to 6.9 and 7.6 % of the bacterial communities after incubation respectively without or with added <sup>13</sup>C-hexadecane (Table S2). Similarly, the abundance of *Sulfurospirillum*-related sequences almost tripled from 1.3 % of the community before incubations to 3.7 and 4.2 % of the communities after incubation respectively without or with added <sup>13</sup>C-hexadecane (Table S2). The majority of the *Geobacter* sequences of this study were related to Fe<sup>III</sup>-reducing *Geobacter psychrophilus* strain P35<sup>[56]</sup> and to *Geobacter luticola*,<sup>[57]</sup> whereas the *Sulfurospirillum* sequences of this study were related to As<sup>V</sup> reducing *Sulfurospirillum deleyianum* strain DSM 6946.<sup>[58]</sup> In addition, *Geothrix*-related sequences were enriched during incubations (Table S2), with the majority of them related to Fe<sup>III</sup> reducing *Geothrix fermentans* strain H5.<sup>[59]</sup>

#### DNA-SIP fractionation of <sup>13</sup>C-hexadecane amended microcosms

DNA-SIP and fractionation was carried out for the <sup>13</sup>C-hexadecane amended microcosms, after the 8-week incubation. If <sup>13</sup>C had not been incorporated into the DNA backbone of the active members of the microbial community, all DNA fractions would contain <sup>12</sup>C-DNA, producing the same DGGE pattern. However, DNA-SIP fractionation followed by DGGE analysis of the PCR amplified DNA fractions, showed clearly that the first few fractions, which correspond to the ‘heavy’ <sup>13</sup>C-labelled



**Fig. 3.** Denaturing gradient gel electrophoresis (DGGE) gel of polymerase chain reaction (PCR) amplified DNA stable isotope probing (DNA-SIP) fractions (collected fractions 3 to 10), after incubation of arsenic-rich Cambodian sediment with <sup>13</sup>C-hexadecane.  $t$  = total isolated DNA (before DNA-SIP fractionation).

DNA, had a distinctly different banding pattern to the DGGE patterns of fractions 8 to 10, which correspond to the light DNA fraction (Fig. 3). The banding pattern of fractions 6 and 7 was a mixture of the heavy and light fractions (Fig. 3). Therefore, the different banding patterns between the collected DNA fractions not only indicate the successful <sup>12</sup>C- and <sup>13</sup>C-DNA separation along the density gradient, but also the successful separation of the DNA from the active members of the bacterial community (heavy DNA fractions 3–5). Moreover, the fact the DGGE profile of the total community (before DNA-SIP fractionation) resembles the pattern of the light DNA fractions (8–10), could be an indication that only a few members of the microbial community became enriched in <sup>13</sup>C.

#### Quantification of extractable <sup>13</sup>C-labelled *n*-alkanes and <sup>13</sup>C-incorporation in the PLFAs

As shown in Table 2, the sediment spiked with <sup>13</sup>C-labelled hexadecane before incubations was dominated by <sup>13</sup>C-hexadecane (1100  $\mu\text{g}$  g<sup>-1</sup> of sediment) but also contained low amounts of other <sup>13</sup>C-labelled *n*-alkanes (i.e. C<sub>17</sub>, C<sub>18</sub>, C<sub>19</sub> and C<sub>20</sub>) with concentrations between 1.3 and 66  $\mu\text{g}$  g<sup>-1</sup> of sediment. The background sedimentary content of *n*-alkanes was 3.7  $\mu\text{g}$  g<sup>-1</sup> of sediment (sum of C<sub>20</sub> to C<sub>34</sub>; data not shown). Following anaerobic incubation for 8 weeks, the amount of <sup>13</sup>C-hexadecane had decreased to 380  $\mu\text{g}$  g<sup>-1</sup> of sediment (65 % degraded), whereas the remaining <sup>13</sup>C-labelled *n*-alkanes,

**Table 2.** Amount of  $^{13}\text{C}$ -labelled *n*-alkanes, as determined following extraction from the original sediment supplemented with 20  $\mu\text{L}$   $^{13}\text{C}$ -hexadecane ( $t = 0$ ), as well as from sediment amended with 20  $\mu\text{L}$   $^{13}\text{C}$ -hexadecane after anaerobic incubation for 8 weeks ( $t = 8$  weeks)

| $^{13}\text{C}$ -labelled<br><i>n</i> -alkanes | $t = 0$<br>( $\mu\text{g g}^{-1}$ sediment) | $t = 8$ weeks<br>( $\mu\text{g g}^{-1}$ sediment) | Amount<br>degraded (%) |
|--|---|---|------------------------|
| C <sub>16</sub>                                | 1071  | 376   | 65                     |
| C <sub>17</sub>                                | 9.6   | 4.0   | 58                     |
| C <sub>18</sub>                                | 66  | 34  | 48                     |
| C <sub>19</sub>                                | 1.3   | 0.6   | 54                     |
| C <sub>20</sub>                                | 2.0   | 1.1   | 45                     |

i.e. C<sub>17</sub>, C<sub>18</sub>, C<sub>19</sub> and C<sub>20</sub>, had respectively degraded by 58, 48, 54 and 45 % (Table 2). The loss of 0.72 mg of hexadecane  $\text{g}^{-1}$  of sediment corresponds to 2.6  $\mu\text{moles}$  of hexadecane  $\text{g}^{-1}$  of sediment (Table 1). It was not possible to quantify  $^{13}\text{C}$ -incorporation in the extracted PLFAs (details in the Supplementary material).

## Discussion

### Degradation of hexadecane and kerogen under arsenic-reducing conditions

During the 8-week anoxic incubations,  $^{13}\text{C}$ -hexadecane was degraded as shown by the 65 % decrease in its concentration. Moreover, this degradation was microbially mediated, as shown by the  $^{13}\text{C}$  incorporation into the heavy DNA fractions of the 'sediment +  $^{13}\text{C}$ -hexadecane' treatment (Fig. 3). Previous studies have shown that *n*-alkanes (including *n*-hexadecane) can be degraded anaerobically by nitrate-reducing or sulfate-reducing bacterial isolates and consortia.<sup>[40,60–62]</sup> Moreover, Rowland et al.<sup>[27]</sup> showed marked degradation of *n*-alkanes (C<sub>20</sub> to C<sub>34</sub>) in microcosms established using arsenic rich sediments. In our study, significant levels of respiration linked to reduction of the very low levels of nitrate and sulfate could not be detected during the course of the experiment, and stoichiometric calculations indicated that neither nitrate or sulfate would have been dominant electron acceptors for the electrons that were potentially donated by the degradation of 2.6  $\mu\text{moles}$  of hexadecane (Table 1). From our data, it would seem clear that the microcosms of this study were predominantly  $\text{Fe}^{\text{III}}$ -reducing systems, with other electron acceptors having minor contribution to the electron transfer balance (Fig. 2; Table 1). Interestingly, as  $^{13}\text{C}$ -hexadecane did not stimulate  $\text{Fe}^{\text{III}}$  reduction we hypothesise that the majority of the electrons were donated by the unidentified indigenous OM (TOC 9.7  $\text{mg g}^{-1}$  sediment, Table 1). However, as higher  $\text{As}^{\text{III}}$  release was observed in the  $^{13}\text{C}$ -hexadecane amended microcosms (Fig. 2d), it is feasible that  $\text{As}^{\text{V}}$  was a minor electron sink for  $^{13}\text{C}$ -hexadecane oxidation (see below). Microbial degradation of  $^{13}\text{C}$ -hexadecane coupled to  $\text{Fe}^{\text{III}}$  or  $\text{As}^{\text{V}}$  respiration, as our results suggest, could be feasible but it would constitute a novel mechanism of respiration. Thus, it remains to be tested in enrichment microcosm experiments, using defined media supplemented with hexadecane as the sole electron donor and  $\text{Fe}^{\text{III}}$  or  $\text{As}^{\text{V}}$  as the sole electron acceptor. Moreover, 16S rRNA gene and arsenate respiratory reductase (*arrA*) gene sequencing of the heavy DNA fractions would enable the respective identification of the  $^{13}\text{C}$ -hexadecane-utilising and arsenate-respiring members of the bacterial community, as shown in previous microcosm studies set up with arsenic

rich sediments and amended with other  $^{13}\text{C}$ -labelled substrates.<sup>[25,26,28]</sup> However, sequencing efforts on the labelled fractions in our study were unsuccessful, most probably due to the very low DNA concentration in the heavy DNA fraction retrieved from the  $^{13}\text{C}$ -hexadecane amended sediment. Instead, we have successfully profiled the whole bacterial communities in three samples, using 16S rRNA gene amplicon pyrosequencing. The results indicated that incubation with or without  $^{13}\text{C}$ -hexadecane led to a clear enrichment in sequences related to  $\text{Fe}^{\text{III}}$ -reducing *Geobacter* (and potentially  $\text{As}^{\text{V}}$ -reducing<sup>[63]</sup>) and *Geothrix* genera, as well as known  $\text{As}^{\text{V}}$ -reducing *Sulfurospirillum* species<sup>[58]</sup> (Table S2). Interestingly, a previous microcosm study that was established with the same sediment and supplemented with  $^{13}\text{C}$ -labelled acetate or  $^{13}\text{C}$ -labelled lactate showed that the heavy DNA fractions were also particularly enriched in sequences related to known  $\text{Fe}^{\text{III}}$ -reducing *Geobacter* and  $\text{As}^{\text{V}}$ -respiring *Sulfurospirillum* species, whereas *Geobacter*-associated *arrA* gene sequences were retrieved too.<sup>[26]</sup>

Interestingly, the supplemented  $^{13}\text{C}$ -hexadecane also contained a mixture of other  $^{13}\text{C}$ -labelled *n*-alkanes (C<sub>17</sub>–C<sub>20</sub>), albeit in much lower amounts, and during the anaerobic incubations the shorter length *n*-alkanes were generally degraded to a greater degree than the longer chain *n*-alkanes (Table 2). However, this finding cannot be generalised, because previous studies showed that different bacterial isolates can utilise different long chain *n*-alkanes under aerobic or anaerobic conditions.<sup>[42]</sup> For example, under aerobic conditions *Marinobacter hydrocarbonoclasticus* strain 617 exhibited higher degradation of the C<sub>19</sub>–C<sub>21</sub> than C<sub>16</sub> *n*-alkanes,<sup>[64]</sup> whereas under anaerobic conditions sulfate-reducing strains Hxd3 and Pnd3 grew on *n*-alkanes in the range of C<sub>12</sub>–C<sub>20</sub> and C<sub>14</sub>–C<sub>17</sub> respectively.<sup>[65]</sup> Moreover, a slurry microcosm study showed that lower molecular weight *n*-alkanes (C<sub>11</sub> to C<sub>13</sub>) were more recalcitrant than mid- to high-molecular weight *n*-alkanes under nitrate-reducing conditions, whereas under sulfate-reducing conditions degradation of long-chain (C<sub>32</sub> to C<sub>39</sub>) *n*-alkanes was more extensive.<sup>[66]</sup> Thus, relative degradation rates of different *n*-alkanes are dependent on ecological and environmental conditions, including the structure of the microbial community present and electron acceptor availability.

Although  $^{13}\text{C}$ -hexadecane (and other *n*-alkanes) was clearly degraded during the incubation period by the microbial communities present, microbial degradation of the  $^{13}\text{C}$ -kerogen analogue could not be established in this study. DNA-SIP fractionation was not attempted for the  $^{13}\text{C}$ -kerogen amended microcosms because of very low DNA yields (data not shown), and the extracted PLFAs were too low in order for  $^{13}\text{C}$ -incorporation in the PLFA fraction to be confirmed with confidence. Thus, it appears that kerogen, the non-extractable fraction of the OM, was non-degradable under anaerobic conditions and over the time periods that we monitored. Future studies could explore again the anaerobic biodegradation of this type of OM, but over longer time scales, as this is something that has been stipulated but has not yet been confirmed conclusively.<sup>[45]</sup>

### Effect of *n*-alkanes and kerogen on $\text{As}^{\text{III}}$ mobilisation

The effect of the addition of  $^{13}\text{C}$ -labelled hexadecane and  $^{13}\text{C}$ -labelled kerogen on  $\text{As}^{\text{III}}$  mobilisation was investigated in anaerobic microcosms that were set up using arsenic-rich Cambodian sediment. The results indicated significant  $\text{Fe}^{\text{III}}$  reduction and low  $\text{As}^{\text{III}}$  mobilisation in all biotic microcosms of



this study ('sediment only' or amended with  $^{13}\text{C}$ -labelled hexadecane or kerogen) but not in the sterile–abiotic microcosms (Fig. 2), confirming that these processes are biologically driven, as shown previously.<sup>[19,20,22]</sup>

The  $^{13}\text{C}$ -hexadecane-amended microcosms showed similar levels of  $\text{Fe}^{\text{III}}$  reduction and a noticeable increase in  $\text{As}^{\text{III}}$  release into the porewaters, in comparison to the 'sediment only' incubations (Fig. 2). Moreover, during incubations of the microcosms supplemented with  $^{13}\text{C}$ -hexadecane, there was a clear microbial degradation of the added  $^{13}\text{C}$ -hexadecane during the incubation (65 % of the supplied  $^{13}\text{C}$ -hexadecane was degraded), as discussed above. However, because there was some variability in the measured  $\text{As}^{\text{III}}$  values (noted by the error bars, representing standard deviation from the mean, Fig. 2d), it is difficult to conclude unequivocally whether the observed increase in  $\text{As}^{\text{III}}$  release reflects an experimental error due to sediment heterogeneity or is a result of stimulated microbial metabolic activity, due to the presence of additional bioavailable OM (the supplemented *n*-alkanes). Overall, the extent of  $\text{Fe}^{\text{III}}$  reduction and  $\text{As}^{\text{III}}$  release in these microcosms, measuring up to  $7.84 \text{ mg Fe}^{\text{II}} \text{ g}^{-1}$  of sediment and up to  $66 \text{ ng As}^{\text{III}} \text{ g}^{-1}$  sediment, was comparable with the results of a previous microcosm study that was set up with sediments from the same area, but from a deeper depth (28 m, sample SYII28; Rowland et al.<sup>[22]</sup>). In the latter study, the addition of acetate did not significantly enhance  $\text{Fe}^{\text{III}}$  reduction and  $\text{As}^{\text{III}}$  release in comparison to the non-amended sediment, and up to  $7 \text{ mg Fe}^{\text{II}}$  and  $46 \text{ ng As}^{\text{III}} \text{ g}^{-1}$  sediment were detected after 4 weeks of incubation. However, Rowland et al.<sup>[22]</sup> observed much higher  $\text{As}^{\text{III}}$  release (up to  $400 \text{ ng g}^{-1}$  of sediment) when microcosms were set up with sediment from 9-m depth and amended with acetate. This was attributed to the presence of an appropriate organic reductant in the deeper sediments, albeit at low concentrations, but its absence at shallower depths. Taking into account that  $\text{As}^{\text{III}}$  may be reduced but remain sorbed and not partition into the aqueous phase, the release of  $\text{As}^{\text{III}}$  in the porewaters does not depend only on the nature of bioavailable OM but also on the specific geochemistry and mineralogy. Thus, future studies on the effect of organic availability on the extent of microbially driven  $\text{As}^{\text{V}}$  mobilisation should also determine the speciation of arsenic in the solid phase using XANES, as it was done previously.<sup>[19]</sup>

In contrast to  $^{13}\text{C}$ -hexadecane amended microcosms, amendment with  $^{13}\text{C}$ -kerogen did not result in increased  $\text{As}^{\text{III}}$  release. This could be attributed to the fact that only small amounts of  $^{13}\text{C}$ -kerogen was supplemented in comparison to background concentrations of OM, and to the recalcitrant nature of this OM under anoxic conditions. In fact, it has been noted that sediments of older age have higher proportions of recalcitrant OM (which has not been biodegraded), something which could explain the reverse relationship between (microbially mediated)  $\text{As}^{\text{III}}$  release and sediment age.<sup>[67]</sup> However, it is not clear why in this study  $^{13}\text{C}$ -kerogen amended microcosms exhibited lower  $\text{Fe}^{\text{III}}$  reduction and  $\text{As}^{\text{III}}$  release than the non-amended microcosms (Fig. 2). This discrepancy is most likely due to the heterogeneous nature of the sediments used, for example with respect to background (Fe–As) mineralogy, supported by the higher initial  $\text{Fe}^{\text{II}}$  levels noted in kerogen amended microcosms.

In summary, this study demonstrated that natural microbial communities can utilise long chain *n*-alkanes under anaerobic conditions, and the addition of  $^{13}\text{C}$ -hexadecane appeared to have a minor but potentially significant effect on  $\text{As}^{\text{III}}$  release from the arsenic-rich sediments used. In contrast,  $^{13}\text{C}$ -kerogen

appeared not to be utilised over the time scale of our study, most likely due to the recalcitrant nature of this substrate or due to the presence of other bioavailable sedimentary OM in much higher amounts than the added  $^{13}\text{C}$ -kerogen. Further studies are required to clarify with confidence whether *n*-alkanes can be utilised in situ by arsenic respiring microorganisms and, if so, whether these organic substrates may play a critical role in the mobilisation of arsenic in shallow reducing aquifers across the globe.

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